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Research article

Seasonal variations coupled with elevation gradient drives significant changes in eco-physiological and biogeochemical traits of a high altitude evergreen broadleaf shrub, *Rhododendron anthopogon*

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ABSTRACT

Higher elevations and, early as well as late phase of growing season are expected to be more stressful for plants in high altitudes. The present study was carried out on *Rhododendron anthopogon* D. Don, an evergreen shrub of Himalaya to understand variation in eco-physiological and biogeochemical traits due to combined effect of elevation gradient and growing season. We conducted our study at Rohtang, India (32°22′04″ N 77°15′17″ E) and undertook random sampling of leaves at four elevations (3200 m, 3600 m, 4000 m and 4250 m), and three time periods (late June, early August and late September) during growing season. We assessed 12 eco-physiological and biogeochemical variables and analysed results through ANOVA and multivariate analysis. It was found that leaf relative water content, nitrogen percentage (N%), carbon/nitrogen ratio (C/N ratio), total chlorophyll, malondialdehyde equivalents and proline content varied along two gradients (factors) with their interaction being statistically significant. Variance partitioning analysis of studied traits revealed that both factors contribute significantly, with 'season' component ranging between 55.75 % and 94.03 % for most of the parameters, whereas, 'elevation' component contributed more for leaf area, N% and C/N ratio (48.08 %–75.03 %). Our results suggest that eco-physiology of *R. anthopogon* is significantly influenced by interaction of seasonal variations coupled with elevation gradient. The study highlights the importance of examining both seasonal and elevational gradients in understanding plant adaptation strategies. Overall, our findings revealed that plasticity in eco-physiological and biogeochemical traits underline the wide distribution of *R. anthopogon* in the high altitudes.

1. Introduction

Gradients of environmental stress are important drivers of variation in plants (Laliberté et al., 2014) and the stress gradient hypothesis assumes higher elevations to be more stressful for plants (Callaway et al., 2002). Reduction in atmospheric temperature and pressure along with increasing solar radiation, higher fraction of UV-B radiation, are primarily associated with increasing elevations (Korner, 2007). The higher elevations are characterised by a wide range of environmental conditions such as low mean annual temperature, diurnal thermal fluctuations, high wind velocity, low partial pressure of gases, high solar radiation intensities, more snow-cover duration *etc*., which limit the plant fitness (Rajsnerová et al., 2015). For instance, declining temperature with increasing elevation evidently causes reduction in plant growth rate (Weih and Karlsson, 2001). Further, intensity of solar radiations increases with altitude and could be regarded an advantage for high altitude plants as far as enhancement in photosynthetic efficiency is concerned (Terashima et al., 2011), but this may also have adverse effects due to photo-oxidation of chlorophyll caused by intense light conditions (Taiz and Zeiger, 2010). Hence, plants may have to invest in accessory and photo-protective pigments to protect themselves as well as utilize high solar radiations. Also, decreased partial pressure of gases and increased air dryness at higher elevations tends to increase leaf-to-air vapour pressure deficit which in turn increases water demand to carry out carbon fixation (Wang et al., 2017a). Overall, dehydrating conditions may be encountered by plants which necessitates the formation of osmoprotectants to tolerate desiccation (Gale, 2004). Besides climatic limitations, soil physico-chemical properties may also vary

Abbreviations: C/N ratio, Carbon/nitrogen ratio; LA, Leaf area; LDMC, Leaf dry matter content; MDA, Malondialdehyde equivalents; N%, Nitrogen percentage; RWC, Relative water content; SLA, Specific Leaf Area; Total Chl, Total chlorophyll content; TPC, Total phenolics content; TSS, Total soluble sugars

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with increasing elevations and may have an impact on plant growth (Ahmad et al., 2018; Ordonez et al., 2009). High altitude plants grow for a very short period which show alterations in air temperature, photoperiod and water availability affecting the growth and distribution of plant species (Kofidis et al., 2003). Various mild to severe environmental stress factors (hereafter referred to as 'multiple stress factors') corresponding to elevation gradient and growing season respectively in alpine environments overlap and hence may influence the plant functioning. High altitude plants serve as models for elucidation of abiotic stress tolerance pathways in plants (Bhardwaj et al., 2013; Kumar, 2016). Knowledge of phenological stage of these plants along with specific elevations at which they grow are vital for such contributions as these conditions control the production of secondary metabolites (Zhu et al., 2016), which in turn influence the adaptation strategies of plants to survive the extremes. Thus, an understanding of inherent adaptation strategies of high altitude plants to peculiar harsh environments is of immense use.

To cope up with "multiple stress factors" of high altitudes, plants must have equipped themselves by evolving plasticity in various morphological, physiological and biogeochemical traits enabling their survival during heterogeneous suboptimal environments (Coldren, 2013; Valladares et al., 2006). For instance, morphological traits such as leaf area, leaf thickness and leaf shape generally show consistent plastic responses with increasing altitude (Kofidis et al., 2007). In alpine environments, plant species more often grow for a very short period after breaking dormancy, and consume most of their assimilated nutrient reserves for protection against environmental cues resulting in slow growth, which usually results in a higher leaf nitrogen content (N %) and lower carbon nitrogen ratio (C/N ratio) (Zhang et al., 2012). Similarly, accumulation of soluble sugars such as raffinose and glucose, proline content and glycine-betaines has been primarily associated with adaptive response to low temperatures and water deficits (Mahajan and Tuteja, 2005). Further, it has also been reported that plasticity in morphological traits have higher energy cost than physiological traits (Li et al., 2016). Both morphological and physiological traits may be altered in plants depending on the long or short-term requirement. For instance, changes in leaf area (LA) and specific leaf area (SLA) are long-term investment for enhanced photosynthetic activity, whereas, adjustments in chlorophyll content and osmoprotectants level (soluble sugars, proline etc.) are inherently low-cost strategies in plants for temperature fluctuations and intense solar radiations (Poorter et al., 2006). Overall, an analysis of environmentally induced trait variation in natural spatio-temporal gradients is necessary to understand the drivers of trait variation. This will also be helpful in elucidating the extent to which plants will respond or be buffered from changing climate (Li et al., 2016).

Leaf is a vegetative organ which is known to exhibit trait plasticity in response to environmental heterogeneity (Xu et al., 2009) and hence, is crucial to study the resource trade-off between growth and environmental protection. Variations and trends in and among leaf traits may depend on changes due to "multiple stress factors" of environmental gradients. Thus, studying variation in leaf eco-physiological and biogeochemical parameters may be helpful to understand the adaptive mechanisms of plants in high altitude regions where environmental factors change over relatively small scales (Byars et al., 2007). Different adaptations in leaves in response to various abiotic stress factors, reported so far, include morphometric variations, accumulation of compatible solutes, pigment accumulation, high secondary metabolites *etc*. (Ma et al., 2015). For instance, leaf dry matter content (LDMC) and SLA are responsive traits of leaves reflecting the structural adaptations to abiotic factors (Hu et al., 2015a). Leaf relative water content (RWC) is considered as a meaningful index of plant water status, and one of the most limiting environmental factors that influence dynamics of plants, as composition, structure and metabolism of the plant is dependent on leaf water status (Hayatu et al., 2014).

Effects of either increasing elevation (Takahashi and Matsuki, 2017; Wang et al., 2017a,b; Zhou et al., 2017) or growing period (Jaeger et al., 1999; Shin et al., 2015) on eco-physiology of plants have been reported by many authors, but its response to combined effect of elevation and growing season is yet to be understood well and, to the best of our knowledge, very few studies were found in literature (Bernal et al., 2013; Kofidis et al., 2003) in this regard. Moreover, the majority of studies emphasizing on either effect (elevation or growing period) have focused on herbaceous species (Hacker and Neuner, 2006), with some studies also on evergreen species such as *Picea* sp. (Angelcheva et al., 2014), *Polylepis* sp. (García-Plazaola et al., 2015) *etc*.

Despite survival in harsh climatic conditions, broadleaf evergreens of high altitudes have received far less attention in context to combinatorial effect of elevation and season, which limits our general understanding of plant adaptation strategies. We focused our study on *Rhododendron anthopogon* D. Don. It is a broadleaf evergreen woody shrub with schlerophyllous leaves, belonging to family *Ericaceae*. It is endemic to western Himalaya and occurs at elevations from treeline zone to up to 4400 m amsl. In our study site, it was found to occur along a wide elevational gradient (*i.e*. from 3200 to 4250 m). *R. anthopogon* is also known to exhibit several medicinal properties such as antibiotic, antiviral, antimycobacterial, anti-inflammatory, antiproliferative *etc*. due to presence of several bioactive molecules (Popescu and Kopp, 2013).

The variations in differences in mean and minimum temperatures at high altitudes of western Himalaya with growing season determine a clear seasonality to which the plant must respond. Therefore, this study was undertaken to understand the combined effect of elevation and growing season on a broadleaf evergreen woody species, *R.anthopogon*. The following objectives were specified: (1) to study the eco-physiology of *R.anthopogon vis-a-vis* environmental gradients of elevation and different time periods of growing season; (2) to elucidate magnitude or contribution of these factors to overall variation of the eco-physiology (bio-chemical/physiological changes associated in response to factors) of *R.anthopogon*. This study is an attempt to understand the variations in morphological, biogeochemical and physiological leaf traits along elevation during different time periods of growing season in a broadleaf evergreen species of Himalaya.

2. Materials and methods

2.1. Study area and plant sampling

The present study was carried out at Rohtang (32°22′04″ N 77°15′17″ E) in Pir Panjal Range of western Himalaya, India (Fig. 1). This region is characterised by low mean annual temperatures and precipitation mostly in the form of snow. In this study, the sampling sites were established at four elevations along an altitudinal transect (3200 m, 3600 m, 4000 m and 4250 m) and sampling was undertaken for three time periods during growing season (23–26 June, 4–7 August, and 23–26 September 2016; hereafter referred to as 'June', 'August' and 'September' respectively). Leaves were sampled on north facing slope of the mountain (Fig. 2). The growing season at the study site starts in middle of June depending on snow melt, followed by optimum growth conditions from mid of July to mid of August (depicted by peak flowering period of most of the species). September is marked by low temperatures with majority of plants entering into dormancy phase.

The plant sampling was carried out during extended noon period to avoid weather complications. The twelve measured parameters were as follows: RWC, LDMC, LA, SLA, N%, C/N ratio, total chlorophyll and carotenoid content, lipid peroxidation (Malondialdehyde (MDA) equivalents), total phenolics content (TPC), proline content and total soluble sugars (TSS) content. To measure RWC, LA, LDMC and SLA, a total of thirty sun exposed and healthy leaves were randomly collected from different individuals at each sampling site. Leaf age during sampling was kept as uniform as possible by sampling the recently fully expanded mature leaves from uppermost leaf canopy whorl. These

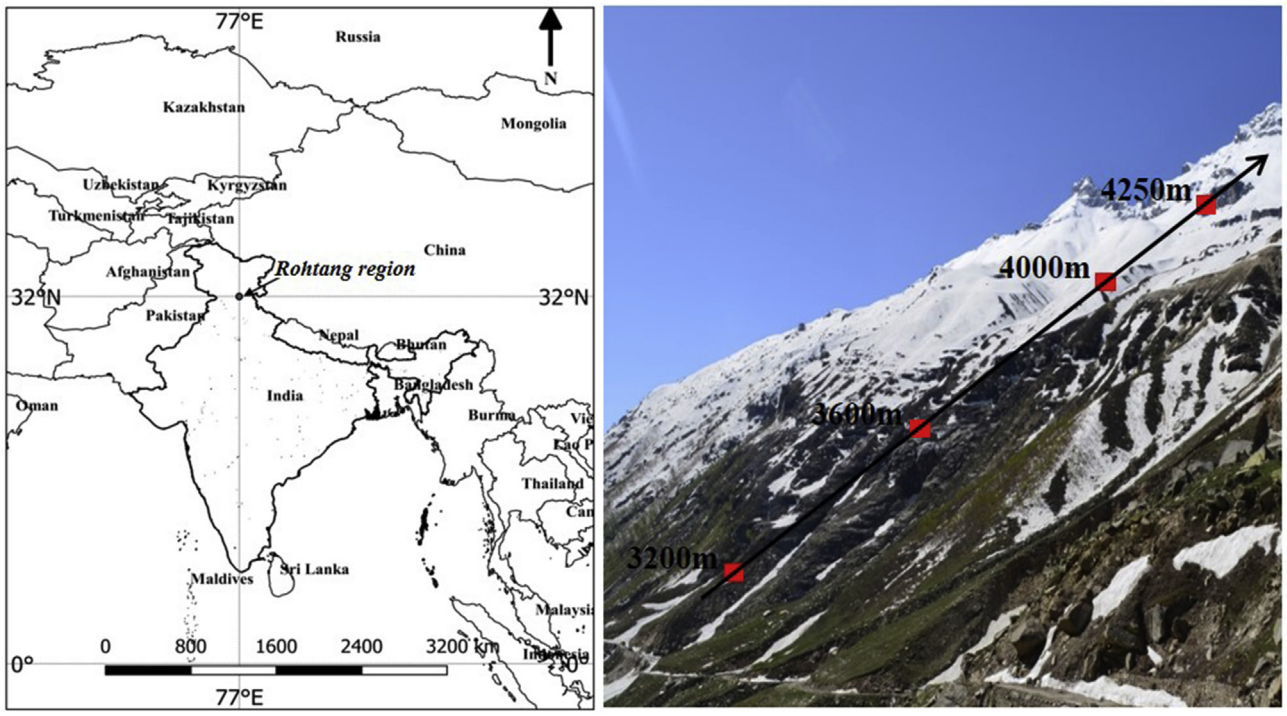

Fig. 1. Location map of study site (Rohtang) in Western Himalaya, India (32°22′04″ N, 77°15′17″ E) (left). Illustration of four different sampling elevations (red rectangles) in the study site (right). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

leaves were used to measure the fresh weight (FW), saturated weight (TW), dry weight (DW) and leaf area so that aforementioned traits could be calculated. After measuring these traits, oven dried leaves were crushed into fine powder and used to perform elemental analysis (N% and C/N ratio). For biochemical estimations, leaves (recently fully expanded, mature, sun exposed and healthy from uppermost leaf whorl) from same population were collected in five replicates and immediately frozen in liquid nitrogen and stored at −80 °C until processed. The sampling was performed in a completely randomized design. Repeated sampling at different elevations was performed in the same populations so that results are comparable.

2.2. Reagents

Chemicals used in various estimations such as toluene, ninhydrin, glacial acetic acid, folin-ciocalteu reagent, methanol, sodium carbonate, anthrone, tri-chloroacetic acid (TCA), thio-barbituaric acid (TBA), ethanol, sulphuric acid and acetone were purchased from HIMEDIA (India). L-Proline, D-glucose and gallic acid standard were obtained from Sigma Aldrich (USA). Chemicals for soil analysis such as sodium hexa-metaphosphate, potassium dichromate, potassium permanganate, ammonium molybdate, potassium antimony tartarate, ethylene diamine tetra-acetic acid (EDTA), ammonium nitrate, ammonium fluoride, potassium dihydrogen phosphate, acetic acid, nitric acid, methyl red, bromocresol green, boric acid and sodium hydroxide were purchased from CDH (India).

2.3. Sample analysis

2.3.1. RWC, LDMC, LA and SLA

We measured four plant functional traits that reflect plant ecological strategies *i.e.* RWC, LDMC, LA and SLA. For RWC, LDMC and SLA, ten leaves were collected from at least 10 individuals, with total of three replicates (10 leaves x 3 replicate = 30 leaves) at each sampling site and each sampling period of growing season. FW was taken immediately after leaves were collected using portable weighing balance (Kern, 572, Balingen, Germany). Leaf samples were brought to the laboratory and kept in moist paper towels (saturated with deionized water) at 4 °C overnight. Leaf SW was measured using a precision balance (Mettler Toledo, ME104, Germany). Leaves were scanned and leaf area was calculated using 'Image J' software (Schneider et al., 2012). For DW, leaves were kept in a hot air oven (Narang Scientific, NSW 123, Ambala, India) at 65 °C for 72 h. RWC was calculated as RWC (%) = (FW-DW)/SW-DW)*100 (Turner, 1981). LDMC and SLA were estimated following the method prescribed by Perez Harguindeguy et al. (2013). LDMC ($g.g^{-1}$) is measured as ratio between leaf dry mass and leaf fresh

Fig. 2. Habitat of *Rhododendron anthopogon* (left) and plant in flowering stage (right).

mass i.e. DW/FW. SLA ($cm^2.g^{-1}$) is measured as ratio of leaf area and leaf dry mass.

2.3.2. Elemental analysis

Oven dried leaves were crushed in mortar and pestle into fine powdered form and processed for analysis of elemental N% and C/N ratio using CHNS analyser Vario MICRO CUBE (ElementarAnalysensysteme GmbH, Germany).

2.3.3. Biochemical analysis

2.3.3.1. Estimation of photosynthetic pigments. Total chlorophyll and carotenoid content was estimated using method described by Arnon (1949). Briefly, 100 mg of frozen grounded tissue was mixed in 80 % pre-chilled acetone. The crude extract was kept overnight in dark at 4 °C. Then this extract was centrifuged at 3000 rpm at 4 °C for 15 min. Supernatant was transferred to fresh falcon tube and absorbance was taken at 663 nm, 645 nm and 480 nm for the estimation of chlorophyll and carotenoid content. 80 % acetone was used as blank control. The chlorophyll concentrations were calculated as follows: Total chlorophyll (a + b) ($mg.g^{-1}$) = (8.02 × A_{663} + 20.20 × A_{645}) x V/1000 × W; where V = Volume of extract (ml) and W = Weight of the leaf tissue (g). Carotenoid content was calculated using the equation provided by (Kirk and Allen, 1965) as follows: A_{480} + (0.114 × A_{663} - 0.638 × A_{645}). All spectrophotometric measurements were carried out using a UV–visible microplate reader (Thermo scientific MULTISKAN GO with cuvette, Finland).

2.3.3.2. Lipid peroxidation. Lipid peroxidation was assayed in terms of malondialdehyde (MDA) equivalents according to Hodges et al. (1999). This assay is based on the reaction of a chromogenic reagent, 2-thiobarbituric acid, with MDA at 25 °C forming chromophore complex with maximum absorption at 532 nm. Frozen grounded leaf sample (100 mg) was homogenized in 1 ml of 0.1 % (w/v) TCA and centrifuged at 12000 g for 15 min at 4 °C. The supernatant (0.4 ml) was mixed with 1 ml of 0.5 % TBA prepared in 20 % TCA and heated at 90 °C for 15 min. The reaction was stopped in ice bath immediately. After cooling, reaction mixture was centrifuged at 12000 g for 15 min at 4 °C and 0.2 ml supernatant was used to determine the absorbance at 532 and 600 nm respectively. MDA equivalent (μmoles.g^{-1} FW) was calculated as: [A x D.F x Vt x 1000]/[E x FW x path length]; where A = ($Absorbance_{532}$ – $Absorbance_{600}$), D.F = Dilution factor, Vt = Volume used (ml), E = Extinction coefficient of MDA (155 mM^{-1} cm^{-1}).

2.3.3.3. Determination of total phenolics content (TPC). TPC was estimated colorimetrically following the method described by Ainsworth and Gillespie (2007). Briefly, 20 mg frozen grounded leaf tissue was homogenized with 2 ml of chilled 95 % methanol in an ice-cold mortar and pestle. Samples were incubated at room temperature for 48 h in dark. Crude extract was centrifuged at 11000 rpm for 5 min at room temperature. Supernatant was collected in fresh tube. For experimental replicates, 100 μl supernatant was dispensed in three tubes and 200 μl 10 % Folin- Ciocalteu reagent was added. Following this, 800 μl Sodium carbonate (700 mM) was added and assay tubes were incubated at room temperature for 2 h. 200 μl assay mixture was added to 96 well microplate and absorbance was taken at 765 nm in a microplate reader. Gallic acid was used as standard to form calibration curve. TPC ($mg.g^{-1}$) is calculated as: TPC = C x V/W where C is gallic acid equivalents ($mg.ml^{-1}$) measured using the regression equation between gallic acid standard and $Absorbance_{765}$, V is volume of crude extract used (ml) and W is the weight of tissue (g).

2.3.3.4. Proline assay. Proline was estimated colorimetrically based on formation of proline-acid ninhydrin complex according to Bates et al. (1973). L-proline was used as standard. Frozen grounded leaf tissue (200 mg) was crushed in liquid nitrogen and homogenized with 4 ml of 3 % aqueous sulpho-salicylic acid. Homogenate was centrifuged at 10,000 rpm (Eppendorf 5804R, Germany) for 15 min at 4 °C. Supernatant was used for estimation of proline content. The reaction mixture consisted of 2 ml supernatant, 2 ml acid ninhydrin and 2 ml of glacial acetic acid in teflon capped glass tubes, which was then boiled at 95 °C for 1 h. The reaction was terminated in an ice bath followed by extraction with 4 ml of toluene. Reaction tubes were stirred vigorously for 15–20 s and kept undisturbed for 30 min at room temperature. Absorbance of chromophore containing toluene formed in upper phase was taken and read at 520 nm in a micro-plate reader. The proline concentration was determined from a standard curve and calculated on a fresh weight basis as: "μmoles proline per gram tissue of fresh weight material = [(μg proline.ml^{-1}) x (ml) toluene x (ml) sulpho-salicylic acid]/(115.5 μg μmol x sample (g)).

2.3.3.5. Total soluble sugars (TSS). TSS was estimated following the anthrone method described by Yemm and Willis (1954) with some modifications. Frozen leaf tissue (50 mg) was homogenized with 5 ml of chilled 80 % ethanol in an ice-cold mortar and pestle. Ethanolic crude extract was centrifuged at 1500 g for 15 min at 4 °C. Supernatant (100 μl) was mixed with 2 ml of chilled 0.2 % anthrone reagent (0.2 g anthrone in 100 ml of 72 % sulphuric acid) and vortexed thoroughly for 20 s. Assay tubes were boiled in water bath at 95 °C for 10 min. The reaction was terminated in an ice bath. Reaction tubes were stirred vigorously for 15–20 s and absorbance was taken at 620 nm in microplate reader. TSS was determined using a calibration curve drawn for glucose standard solutions and expressed as $mg.g^{-1}$ fresh weight.

2.3.4. Soil physico-chemical analysis

Five surface soil samples (0–15 cm depth) were taken at each site along elevation gradient for soil physico-chemical analysis. The samples were cleared of pebbles, stones, surface litter and fine roots. Further, soils were air dried and sieved to get 2 mm fraction. The soil samples were analysed for pH and electrical conductivity. Percentage soil moisture was calculated as (FW_{soil} – DW_{soil})/FW_{soil} multiplied by 100. Particle size distribution (soil texture) was measured using the Bouyoucos hydrometer method (Gee and Bauder, 1986). Percentage soil organic matter was calculated by multiplying percent organic carbon (determined by the acid dichromate digestion method (Walkley and Black, 1934) by a factor of 1.724. Available nitrogen was determined by Kjeldahl digestion followed by ammonium distillation and titrimetric determinations (Subbiah and Asiza, 1956) whereas available phosphorus and potassium were measured by the methods prescribed by Buondonno et al. (1992).

2.4. Statistical analysis

The mean ± standard deviation was calculated from three-five replicates per variable. To compare soil physico-chemical characteristics between elevations, one-way ANOVA followed by *Tukey* HSD *post hoc* ($P < 0.05$) test was used. A two-way *ANOVA* was performed on variables in order to evaluate the effect of factors (sampling time and elevation) and their interaction (sampling time *vs.* elevation) on response variables followed by *Tukey* HSD *post hoc* test to identify the values with significant differences. Differences between the values were considered significant at $P < 0.05$. All analyses were performed with R statistical software package (R Development core team 2016). Variance partitioning was conducted to estimate variance due to elevation and season independently for each variable. Principal Component Analysis (PCA) was used to provide an overview of the role of season and elevation in the studied variables and to calculate direction and strength of each variable. Variance partitioning analysis was performed using 'lme4' package with crossed factors (Bates et al., 2015) and PCA was performed using 'vegan' package (Oksanen et al., 2018).

Table 1
Soil characteristics of sampling sites along elevation gradients.

Parameter	3200 m	3600 m	4000 m	4250 m
Soil pH	5.384 ± 0.186	5.326 ± 0.190	5.25 ± 0.153	5.338 ± 0.160
Electrical conductivity (μS.cm^{-1})	68.3 ± 12.43	74.44 ± 18.50	71.98 ± 18.04	67.84 ± 19.36
Soil moisture (%)	15.53 ± 4.553^{a}	30.65 ± 7.857^{b}	35.34 ± 3.636^{b}	29.69 ± 3.043^{b}
Organic matter (%)	1.199 ± 0.101	1.309 ± 0.240	1.364 ± 0.227	1.020 ± 0.227
Soil texture	Sandy loam	Loam	Loam	Loam
Available N (μg.g^{-1})	165.2 ± 75.57	243.6 ± 78.21	252 ± 53.34	191.8 ± 79.90
Available P (μg.g^{-1})	430.7 ± 222.4^{a}	447.1 ± 143.0^{a}	117.7 ± 21.36^{b}	109.6 ± 12.41^{b}
Available K (μg.g^{-1})	140.7 ± 20.20	172.8 ± 36.14	122.5 ± 27.01	155.7 ± 40.17

Different alphabets (a, b, c) represent statistically significant values ($P < 0.05$) as determined by *Tukey* HSD *post hoc* test.

3. Results

3.1. Soil attributes

Soil at all the elevations were slightly acidic in nature (pH ranging from 5.11 to 5.58). Soil texture at 3200 m was sandy loam whereas rest of the elevations were loam. Percentage soil organic matter was low for all the elevations ranging from 0.778 % to 1.715 %. There were no significant differences among sites for electrical conductivity (47.2 μS.cm^{-1}–105.1 μS.cm^{-1}), available nitrogen (84 μg.g^{-1}–350 μg.g^{-1}) and available potassium (89.91 μg.g^{-1}–227.1 μg.g^{-1}). Soil characteristics of the sites varied significantly in terms of soil moisture percentage (9.5 %–40 %) and available phosphorus (102.1 μg.g^{-1}–626.8 μg.g^{-1}) with respect to elevation. Soil moisture percentage was significantly lower at 3200 m in comparison to the rest of the elevations. Also, available phosphorus was significantly low at higher elevations in comparison to lower elevations ($P < 0.05$) (Table 1).

3.2. Variation in eco-physiological parameters of R. anthopogon leaves along elevation and seasonal climatic gradients

The twelve eco-physiological variables of *R. anthopogon* that were studied showed different relative sensitivity to the two climatic drivers of variation. RWC varied in range from 70.26 % to 91.17 %, LDMC from 0.391 g.g^{-1} to 0.522 g.g^{-1}, LA from 9.53 cm^2 to 30.97 cm^2 and SLA from 62.67 cm^2.g^{-1} to 86.44 cm^2.g^{-1} (Table 2). N concentration of leaves was estimated between 1.462 % and 2.226 % and C/N ratio from 23.79 to 35.35. Total chlorophyll ranged between 1.173 mg.g^{-1} and 2.114 mg.g^{-1} and carotenoid content between 0.241 mg.g^{-1} and 0.472 mg.g^{-1}. The range of proline content was between 0.534 μmol.g^{-1} and 1.414 μmol.g^{-1}, whereas, TSS was between 25.14 mg.g^{-1} and 35.72 mg.g^{-1}. MDA equivalent in the leaves varied in range of 75.24 μmol.g^{-1} and 150.6 μmol.g^{-1} and TPC in range of 43.86 mg.g^{-1} and 44.53 mg.g^{-1} (Table 3).

Two-way ANOVA showed significant effects of elevation and growing season gradient as well as their interactions on the leaf traits (Table 4). In particular, elevation, season and their interactions exerted a significant effect on leaf RWC and stoichiometric traits such as N% and C/N ratio, and biochemical parameters such as leaf chlorophyll concentration, MDA equivalent and proline content. Leaf RWC significantly increased during the growing season (Fig. 3a) and was higher for upper elevations than lower elevations in both June and September (Table 3). Leaf N% was significantly higher in the upper elevations as compared to the lower elevations and was significantly reduced towards the end of growing season. This decrease was more for the upper elevations (Fig. 3a, Table 2). In contrast, leaf C/N ratio showed the opposite trend in the growing season showing a gradual increase (Fig. 3a). This was higher for the lower elevations and lower for upper elevations (Table 2). The relative amount of total chlorophyll significantly increased along elevation during August (Table 3) and was more during June (in two out of four elevations) and September (in three out of four elevations) (Fig. 3b).

MDA equivalent content was highest in June (but was found decreasing along elevation gradient) and decreased significantly during August–September (Fig. 3b, Table 3). Further, the lowest MDA equivalent content was at highest elevation in June and September. The proline content showed similar results during the growing season but in opposite direction of elevation gradient. Proline accumulation significantly increased along elevation in August and showed the same elevation patterns in September also. Further, it was more during June, dropped to low levels during August and remained constant in concentration afterwards during September.

Trends due to elevation and season were statistically significant for LA, SLA, TPC and carotenoids and therefore are considered as 'main effects' of two-way ANOVA. Here, the interaction of 'Elevation' and 'Season' factors was not significant. But, the factors showed statistical

Table 2
Season and elevational variations in leaf morphological and stoichiometric traits of *R. anthopogon*.

Season	Elevation (m)	RWC (%)	LDMC (g.g^{-1})	LA (cm^2)	SLA (cm^{2}g^{-1})	N (%)	C/N ratio
June	3200	70.26 ± 3.947	0.500 ± 0.018	3.097 ± 0.580^{a}	72.07 ± 4.486	1.554 ± 0.111^{a}	32.11 ± 2.521^{a}
	3600	77.33 ± 12.52	0.519 ± 0.036	2.578 ± 0.207^{ab}	62.67 ± 1.005	1.690 ± 0.114^{a}	29.04 ± 1.903^{ab}
	4000	78.55 ± 0.648	0.519 ± 0.025	1.810 ± 0.051^{bc}	64.04 ± 6.206	1.946 ± 0.121^{bc}	26.11 ± 1.729^{b}
	4250	77.11 ± 1.414	0.501 ± 0.003	1.628 ± 0.144^{c}	64.14 ± 1.096	1.940 ± 0.169^{c}	25.98 ± 2.242^{b}
August	3200	84.82 ± 3.271	0.405 ± 0.005	3.053 ± 0.919^{a}	86.44 ± 6.722	1.576 ± 0.153^{a}	33.15 ± 2.751^{a}
	3600	88.98 ± 1.112	0.400 ± 0.006	2.004 ± 0.117^{b}	77.25 ± 1.920	1.658 ± 0.023^{a}	31.33 ± 0.921^{a}
	4000	85.53 ± 3.898	0.391 ± 0.006	1.213 ± 0.156^{bc}	79.57 ± 2.176	2.226 ± 0.154^{bc}	23.79 ± 0.843^{b}
	4250	79.15 ± 4.514	0.410 ± 0.009	0.953 ± 0.152^{c}	76.86 ± 0.391	2.106 ± 0.053^{c}	24.57 ± 0.496^{b}
September	3200	88.20 ± 3.787	0.490 ± 0.020	2.617 ± 0.169^{a}	71.48 ± 3.235	1.470 ± 0.032	35.35 ± 0.856^{a}
	3600	82.39 ± 1.582	0.508 ± 0.011	1.994 ± 0.084^{ab}	69.06 ± 3.119	1.462 ± 0.093	34.79 ± 2.457^{ac}
	4000	89.19 ± 2.797	0.522 ± 0.009	1.123 ± 0.092^{b}	64.78 ± 1.181	1.616 ± 0.081	31.43 ± 1.498^{b}
	4250	91.17 ± 1.317	0.511 ± 0.012	1.198 ± 0.225^{b}	70.61 ± 3.148	1.682 ± 0.074	30.40 ± 1.169^{c}

Different alphabets (a, b, c) represent statistically significant values ($P < 0.05$) as determined by *Tukey* HSD *post hoc* test.
RWC = Relative water content (mean ± Std dev.; n = 3); LDMC = Leaf dry matter content (mean ± Std dev.; n = 3); LA = Leaf area (mean ± Std dev.; n = 3); SLA = Specific leaf area (mean ± Std dev.; n = 3); N% = Nitrogen percentage (mean ± Std dev.; n = 5) and C/N ratio = Carbon nitrogen ratio (mean ± Std dev.; n = 5).

Table 3
Season and elevational variations in Proline, Total chlorophyll, Carotenoid content, Total phenolics content (TPC), Malondialdehyde equivalents (MDA) and Total soluble sugars (TSS) in *R. anthopogon* (mean ± Std dev.; n = 5).

Season	Elevation (m)	Proline (μmoles.g^{-1})	Total chl (mg.g^{-1})	Carotenoid (mg.g^{-1})	TPC (mg.g^{-1})	MDA (μmoles.g^{-1})	TSS (mg.g^{-1})
June	3200	1.288 ± 0.132^{a}	2.114 ± 0.205^{a}	0.373 ± 0.059	44.41 ± 0.382	150.6 ± 12.12^{a}	29.25 ± 1.178
	3600	0.961 ± 0.021^{b}	1.591 ± 0.395^{b}	0.437 ± 0.085	44.25 ± 0.414	139.6 ± 4.733^{ab}	31.15 ± 2.109
	4000	1.110 ± 0.144^{b}	1.904 ± 0.097^{ab}	0.418 ± 0.057	44.53 ± 0.574	127.9 ± 2.575^{b}	31.38 ± 2.128
	4250	1.414 ± 0.244^{a}	1.361 ± 0.117^{b}	0.472 ± 0.111	44.33 ± 0.330	127.7 ± 7.542^{b}	31.40 ± 1.408
August	3200	0.548 ± 0.076^{a}	1.355 ± 0.094^{a}	0.455 ± 0.039	44.33 ± 0.357	78.09 ± 3.156	25.34 ± 3.096
	3600	0.613 ± 0.054^{a}	1.476 ± 0.069^{ab}	0.457 ± 0.024	44.05 ± 0.237	78.55 ± 4.045	25.42 ± 1.338
	4000	0.698 ± 0.081^{a}	1.625 ± 0.135^{ab}	0.411 ± 0.127	44.25 ± 0.574	75.24 ± 2.329	25.54 ± 1.055
	4250	0.900 ± 0.161^{b}	1.731 ± 0.108^{b}	0.381 ± 0.041	43.98 ± 0.260	81.06 ± 6.078	25.14 ± 2.214
September	3200	0.534 ± 0.064	1.543 ± 0.134^{a}	0.241 ± 0.024^{a}	44.25 ± 0.278	86.44 ± 3.098	32.67 ± 1.744
	3600	0.616 ± 0.055	1.173 ± 0.030^{b}	0.413 ± 0.009^{b}	44.09 ± 0.320	78.66 ± 3.600	33.58 ± 3.978
	4000	0.752 ± 0.064	1.785 ± 0.127^{bc}	0.344 ± 0.128^{a}	43.9 ± 0.161	79.04 ± 6.159	35.72 ± 1.025
	4250	0.760 ± 0.030	1.927 ± 0.015^{c}	0.321 ± 0.032^{a}	43.86 ± 0.192	79.03 ± 11.89	35.67 ± 1.542

Different alphabets (a, b, c) represent statistically significant values ($P < 0.05$) as determined by *Tukey* HSD *post hoc* test.

Table 4
Summary of two-way ANOVA showing the effects of season, elevation and their interaction on morphological, physiological and biogeochemical traits.

Parameter	Effect	Df	TSSq	MSSq	F-value	P-value
RWC (%)	Season	2	918	459	22.14	< 0.001
	Elevation	3	50.8	16.9	0.817	0.497
	Season:Elevation	6	353.5	58.9	2.842	0.031
LA (cm^2)	Season	2	2.103	1.051	8.988	0.0012
	Elevation	3	16.22	5.408	46.231	< 0.001
	Season:Elevation	6	0.527	0.088	0.751	0.614
SLA (cm^2.g^{-1})	Season	2	1348.9	674.5	55.39	< 0.001
	Elevation	3	316.1	105.4	8.654	< 0.001
	Season:Elevation	6	105.9	17.6	1.449	0.237
LDMC (g.g^{-1})	Season	2	0.092	0.046	176.2	< 0.001
	Elevation	3	0.0007	0.0002	1.003	0.408
	Season:Elevation	6	0.0024	0.0004	1.564	0.201
C/N ratio	Season	2	298.9	149.46	47.52	< 0.001
	Elevation	3	491.9	163.96	52.13	< 0.001
	Season:Elevation	6	58.3	9.71	3.087	0.012
N (%)	Season	2	1.16	0.58	49.42	< 0.001
	Elevation	3	1.887	0.629	53.59	< 0.001
	Season:Elevation	6	0.412	0.068	5.85	< 0.001
Total Chl (mg.g^{-1})	Season	2	0.409	0.201	8.071	< 0.001
	Elevation	3	1.055	0.352	14.09	< 0.001
	Season:Elevation	6	2.648	0.441	17.68	< 0.001
Carotenoid (mg.g^{-1})	Season	2	0.123	0.061	11.41	< 0.001
	Elevation	3	0.047	0.016	2.944	0.0422
	Season:Elevation	6	0.074	0.012	2.293	0.0501
Proline (μmoles.g^{-1})	Season	2	3.554	1.776	140.74	< 0.001
	Elevation	3	0.729	0.243	19.258	< 0.001
	Season:Elevation	6	0.397	0.066	5.246	< 0.001
TSS (mg.g^{-1})	Season	2	830.3	415.1	95.74	< 0.001
	Elevation	3	30.1	10	2.313	0.0878
	Season:Elevation	6	21.5	3.6	0.825	0.556
MDA (μmoles.g^{-1})	Season	2	43284	21642	515.9	< 0.001
	Elevation	3	1038	346	8.248	< 0.001
	Season:Elevation	6	1056	176	4.196	0.0018
TPC (mg.g^{-1})	Season	2	1.283	0.641	4.873	0.0118
	Elevation	3	0.63	0.209	1.595	0.02
	Season:Elevation	6	0.482	0.08	0.611	0.72

RWC = Relative water content; LA = Leaf area; SLA = Specific leaf area; LDMC = Leaf dry matter content; C/N ratio = Carbon nitrogen ratio; N % = Nitrogen percentage; Total Chl = Total chlorophyll; TSS = Total soluble sugars; MDA = Malondialdehyde equivalents; TPC = Total phenolics content; Df = Degree of freedom; TSSq = Total sum of squares and MSSq = Mean sum of squares.

significance when both were considered independently (Table 4). In our study, significant decrease in LA corresponding to small size of leaves at higher elevations was found. It was found to be gradually decreasing along different sampling periods of growing season. There was marked increase in SLA during August in comparison to June and September (Fig. 3a). The results were significant for June and August. TPC decreased with increasing elevations, but this was evident in September only. These differences were not statistically significant, however. Carotenoid content decreased during September and this decrease was more at lowest elevation than the upper ones (Fig. 3b). TSS was found to be increasing along elevations (Table 3) and was low during August in comparison to June and September (Fig. 3b). No evidence of significant variations and trends in LDMC was observed along increasing elevation in any of the sampling periods. However, it was found to be low during August in comparison to others.

Principal component analysis of traits showed that 62.2 % of total variance in the whole dataset is explained by first two principal components (PC1 and PC2) (Fig. 4; Table 5). Out of the total variance, PC1 axis explained 33.7 % and major explanatory variables associated with PC1 axis were MDA equivalents, proline content, RWC and SLA. Similarly, the PC2 axis explained 28.5 % of variance and was largely related to N%, C/N ratio, TSS and LDMC (Fig. 4; Table 5). It can be interpreted from the PCA biplot that MDA equivalents and proline content were higher during June. MDA equivalents accumulated more in lower elevations while accumulation of the proline content increased at higher elevations. On the other hand, RWC was lowest in June. SLA and N% were higher during August, with the latter more predominant in higher elevations. TSS and LDMC were lowest in August. C/N ratio was higher during September with opposite trends for N%.

3.3. Proportion of trait variation explained by two gradients

To quantify the amount of variation explained by season and elevation independently, for each variable we carried out variance partitioning analysis. Among the studied eco-physiological parameters, six (RWC, SLA, Proline, TSS, MDA and LDMC) out of twelve parameters were having maximum variance due to season (variance contribution ranged between 55.75 % and 94.03 %), three (LA, N%, C/N ratio) were having maximum variance due to elevation (variance contribution ranged between 48.08 % and 75.03 %), whereas, rest three (Tot Chl, Carotenoids and TPC) were having maximum variance due to other sources (Fig. 5; Table 6). Variance from other sources could be due to variance between samples (biological replicates) and/or due to error.

4. Discussion

Rhododendron anthopogon is particularly interesting, both in terms of its altitudinal distribution and ability to survive the marked environmental variations throughout the year. It is therefore expected to possess appropriate mechanisms to cope up with the prevailing harsh conditions of high altitudes. To this end, we investigated the effect of seasonal variation along an elevational gradient on eco-physiological and biogeochemical traits of this broadleaf evergreen shrub species. Our study suggests significant effect of both the key gradients of high

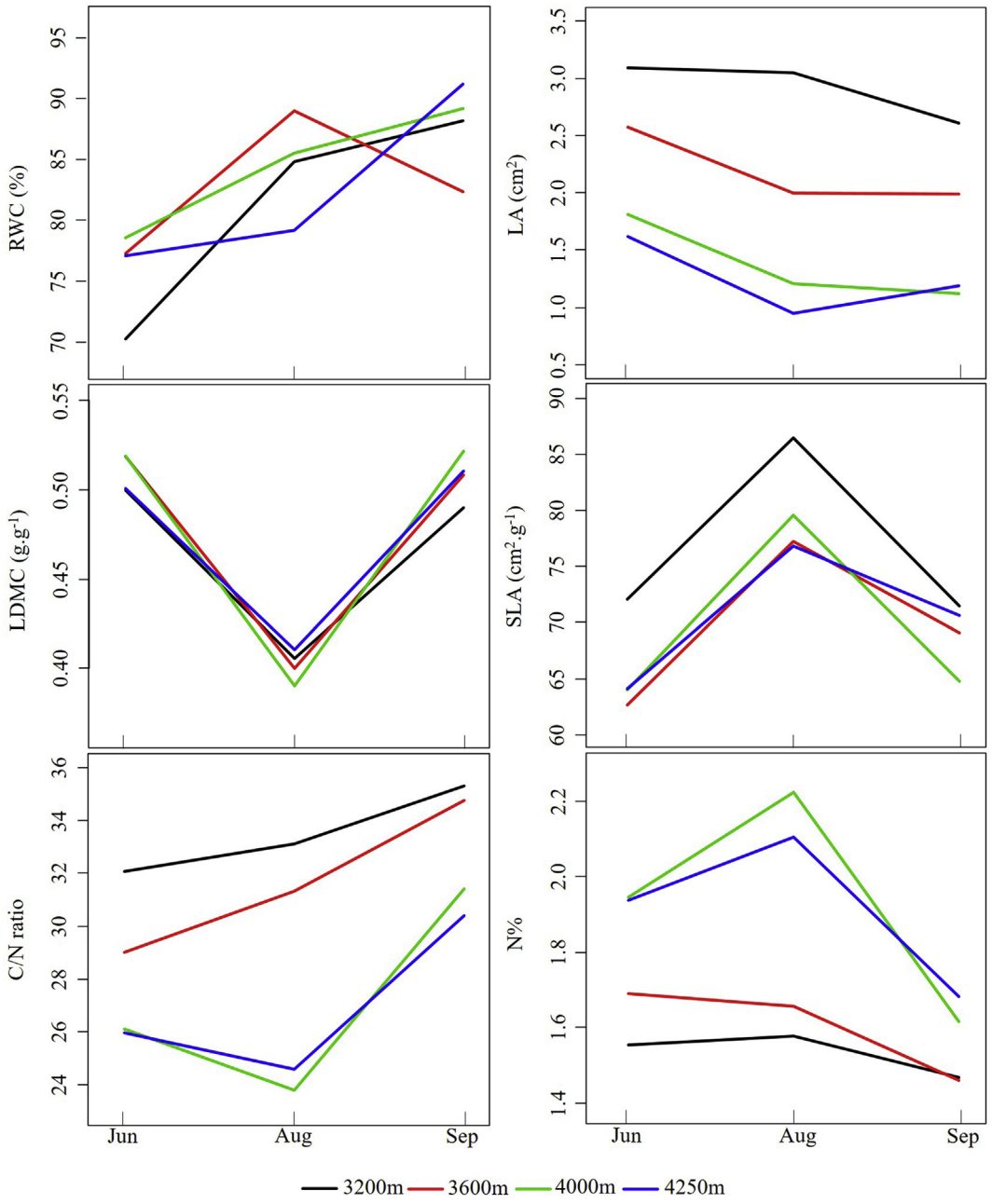

Fig. 3a. Interaction plots (elevation and growing period) for leaf traits. RWC = Relative water content, LDMC = Leaf dry matter content, C/N ratio = Carbon nitrogen ratio, LA = Leaf area, SLA = Specific leaf area, N% = Nitrogen percentage.

altitude environment accounting for variation in studied leaf traits of *R. anthopogon*. Most of the studied leaf traits responded to both elevation and growing season while a few others were accounted to have significant effect of either. We did not observe any significant variation and trends in RWC, LDMC and SLA along the elevation delimiting the contribution of elevation for these leaf traits. Previous attempts to relate these parameters to elevation have obtained mixed results. For instance, Schob et al. (2013) have reported negative relationship of LDMC and SLA with elevation and a positive correlation of RWC with altitude. However, unchanged RWC and SLA along elevation gradients have been reported by Gonzalez et al. (2007) and by Baruch et al. (2017) and Li et al. (2006) respectively. Further, increase in values of RWC from June to September, and lesser LDMC and higher SLA in August in comparison to June and September depicted role of gradients of growing season to be more conclusive in defining the water status and resource use strategy of this particular plant. Higher LDMC in June and September may be attributed to increase in accumulation of solutes (Monson, 2006) and cell wall thickening (Pescador et al., 2016) in response to low temperatures. This may explain why LDMC values increased in these two sampling periods in comparison to lower values during August. As SLA is inversely proportional to LDMC (1/SLA = Tissue density (LDMC) x leaf thickness (Wilson et al., 1999), hence an increase in SLA during August is justified. High SLA and low LDMC values during August suggest high productivity showing preference for rapid production of biomass. On the other hand, in June and September, low SLA and high LDMC in leaves suggested a well-organized nutrient conservation strategy in presumably stressful environments (Garnier et al., 2001). Leaf area was found to be decreasing along elevation in all the seasons and could be an adaptation to lowered air temperatures at higher elevations. This is in agreement with earlier results reported for high altitude plants by Cordell et al. (2012) and Ackerly et al. (2002). Seasonal difference in leaf size was not prominent

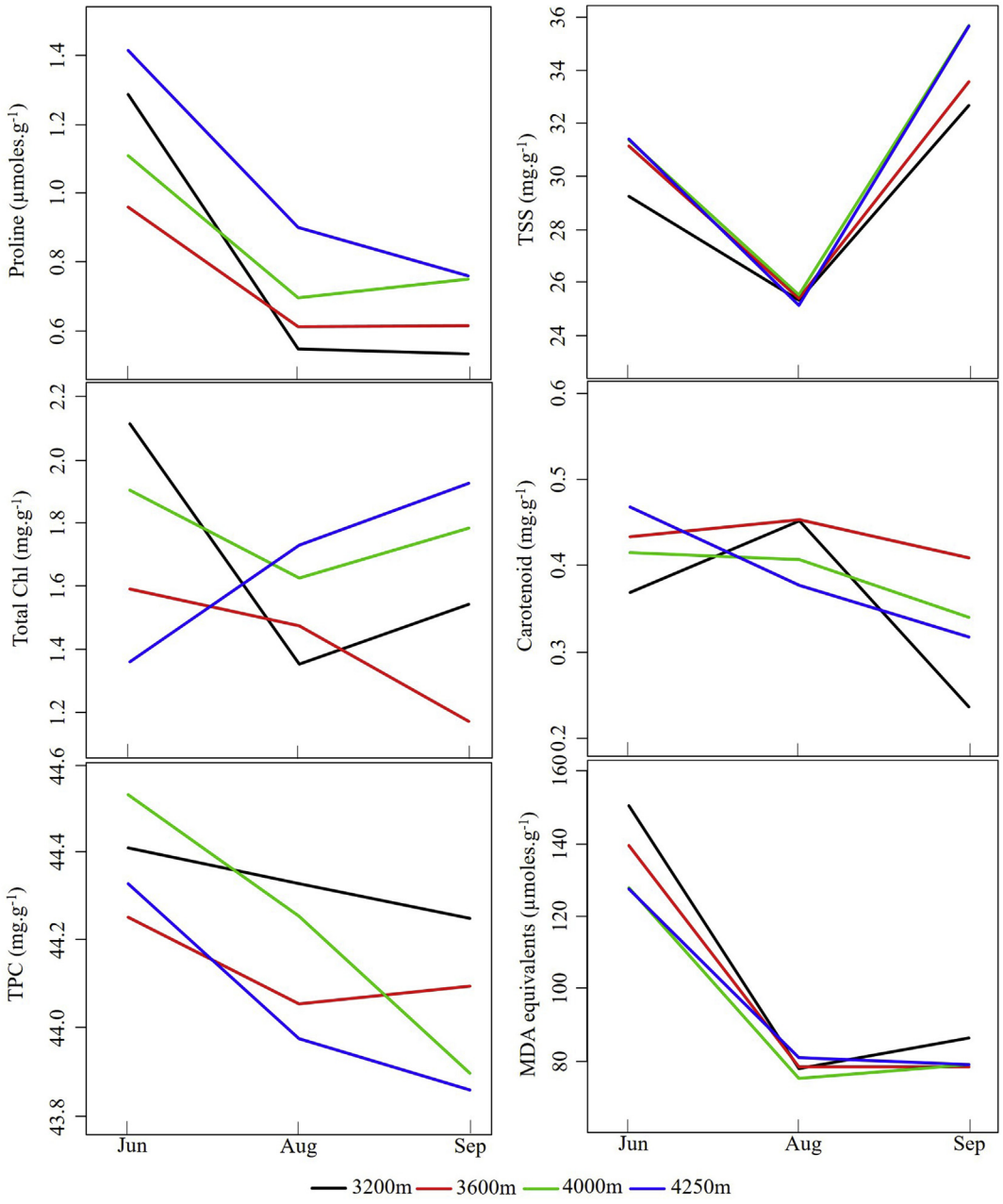

Fig. 3b. Interaction plots (elevation and growing period) for leaf physiological and biogeochemical parameters. Total Chl = Total chlorophyll, TPC = Total phenolics content, MDA = Malondialdehyde equivalents and TSS = Total soluble sugars.

due to the fact that leaf formed once will not enhance its area after it achieves saturation in terms of its growth.

The increase in N% and decrease in C/N ratio along the elevations was found consistent with the results of Wang et al. (2017b). According to (Reich and Oleksyn, 2004), high elevation individuals are predicted to have higher leaf N% with decreasing temperature which ultimately depicts enhanced photosynthetic capacity (Garnier et al., 1999; Qiuhong et al., 2013) and leaf longevity (Korner, 1989) as both are closely coupled to N%. Cold conditions restrict shoot growth more as compared to N uptake by roots, resulting in a higher concentration of nitrogen in leaves (Korner and Larcher, 1988). At upper elevations, N% was significantly higher in August followed by June and September asserting former to be the most favourable period for photosynthetic capacity in *R.anthopogon*. Total chlorophyll is considered to be the key element for photosynthesis and growth (Takashima et al., 2004) and most often regarded as proxy for photosynthetic capacity (Croft et al., 2017). Enhanced total chlorophyll content in leaves along increasing elevation was recorded during time periods of August. Hence, it overall implied that high N% and chlorophyll content helps *R.anthopogon* in sustaining photosynthetic efficiency despite harsh environmental conditions prevailing at higher elevations. Carotenoid content remained more or less stable during the growing periods at all elevations examined.

Lipid peroxidation variations (MDA equivalents) are used to evaluate the status of oxidative damage (Moller et al., 2007; Prasad et al., 2016). MDA is generated as a by-product of reactions involving polyunsaturated fatty acids and reactive oxygen species (ROS). Lower MDA values along the elevation suggest a high anti-oxidative ability of plants avoiding lipid peroxidations reflecting a higher stress resistance (Campos et al., 2003; Hu et al., 2015b). It has been reported that cold susceptible genotypes (probably at low elevations) have more lipid peroxidation rates resulting in high MDA levels in comparison to cold

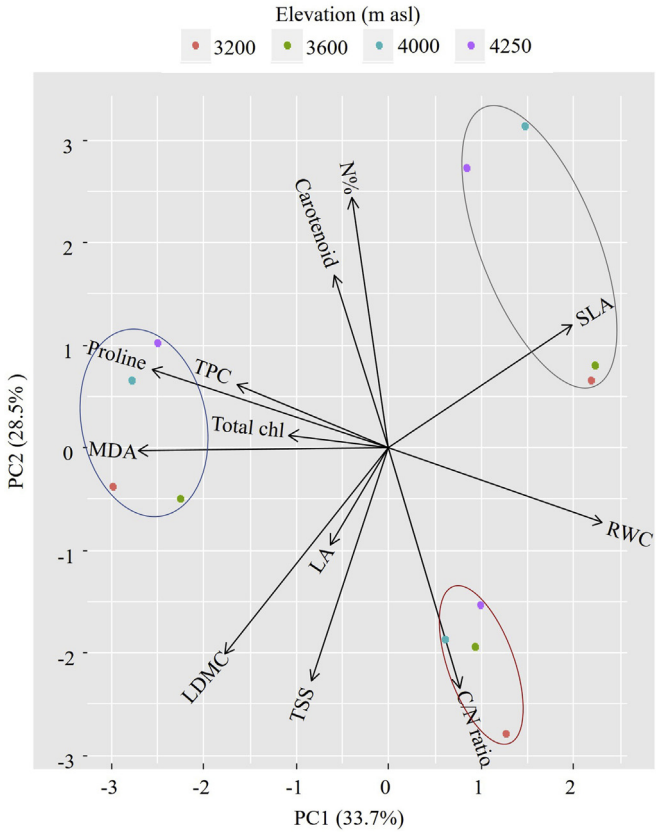

Fig. 4. Ordination by principal component (PCA) analysis of the twelve eco-physiological variables of leaves of *R. anthopogon* from four different altitudes (3200 m, 3600 m, 4000 m, 4250 m) and three different sampling periods i.e. June (highlighted by blue eclipse), August (highlighted by grey eclipse) and September (highlighted by red eclipse). RWC = Relative water content; LA = Leaf area; SLA = Specific leaf area; LDMC = Leaf dry matter content; C/N ratio = Carbon nitrogen ratio; N% = Nitrogen percentage; Total Chl = Total chlorophyll; TSS = Total soluble sugars; MDA = Malondialdehyde equivalents; TPC = Total phenolics content. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 5
Eigenvector scores of eco-physiological variables on the two main principal components (PC) for *R. anthopogon*.

S.no	Parameter	PC1 (33.7%)	PC2 (28.5%)
1	MDA	−0.477	−0.005
2	Proline	−0.45	0.145
3	RWC	0.406	−0.141
4	SLA	0.348	0.230
5	LDMC	−0.312	−0.385
6	TPC	−0.289	0.118
7	TSS	−0.148	−0.437
8	Total Chl	−0.190	0.024
9	LA	−0.112	−0.180
10	C/N ratio	0.135	−0.448
11	N%	−0.070	0.466
12	Carotenoid	−0.103	0.321

Values in parenthesis indicate variance accounted for by each axis.
MDA = Malondialdehyde equivalents; RWC = Relative water content; SLA = Specific leaf area; LDMC = Leaf dry matter content; TPC = Total phenolics content; TSS = Total soluble sugars; Total Chl = Total chlorophyll; LA = Leaf area; C/N ratio = Carbon nitrogen ratio and N% = Nitrogen percentage.

tolerant genotypes (Bonnecarrere et al., 2011). Also, low soil moisture percentage (Noctor et al., 2014) at lower elevations may induce oxidative stress via increased production of ROS resulting in more lipid peroxidation rates. Phenolics shield the plants by absorbing harmful UV-B radiations protecting them from structural damage. Plants in alpine regions are expected to be richer in phenolics in contrast to low altitude species due to more pronounced UV-B radiation stress (Bernal et al., 2013). However, in *R. anthopogon,* decrease in TPC with increasing elevation was observed. Similar observations for number of high altitude species (e.g. *Saxifraga* sp., *Galium* sp., *Plantago lanceolata* (L.)) have been reported by many authors (Rau and Hofmann, 1996; Ziska et al., 1992). This leads to favour the assumption that *R. anthopogon* might have developed a mechanism which is independent of TPC in order to protect itself from high UV-radiations. Increasing concentrations of phenolics are reported as a plant defence mechanism against herbivore attack. With increasing elevation, the herbivore attacks decreases (Carey and Wink, 1994; Hodkinson, 2005), which might account for higher TPC observed at lower elevations.

Proline and TSS accumulation increased along the elevation which act as osmoprotectants and help the plants to survive adverse conditions by enabling them to withstand low temperatures (Zhang et al., 2012). Proline accumulation was found to be more pronounced in June and could be considered a low temperature response of *R.anthopogon.* These results are in line with previously published research showing a similar increase in proline contents (Patton et al., 2007). A significant increase in content of total soluble sugars during September can be linked to the role of carbohydrates to survive low temperatures and raising supercooling capacity of long-lived leaves of the examined evergreen species (Rada et al., 2001). Sugars also have been identified to play an important role in combating drought conditions by acting as osmoprotectant (Lei et al., 2006; O'Brien et al., 2015). In high altitudes, plants face high transpiration pressures because of dehydrating environments (due to high vapour pressure deficit) (Gale, 2004) and low temperatures (inducing drought stress due to decreased water absorption by roots because of low temperature of soil) (Yin et al., 2004). Sugars replace water molecules and establish hydrogen bonds with lipid molecules and protect plant cell membrane thus stabilizing its structure (Ruelland et al., 2009). Therefore, the increased concentration of soluble sugars in *R. anthopogon* at high elevations could be a response to cope with dehydrating conditions as well as low temperatures. Variation in this trait has also implications with respect to future climate which is likely to have frequent unfavourable dry climates. TSS may therefore play a crucial role in persistence of populations of this evergreen species in future.

Overall, we found significant differences in physiological traits indicating their important role in adaptations to the stressful environments of alpine regions in Himalaya in this broadleaf evergreen species. The observed patterns in physiological and biogeochemical traits of *R. anthopogon* demonstrated their roles in providing insights of how plasticity in traits play key role in the species persistence and function. Also, trait variation induced by plasticity in functional traits is presumed to affect the expansion of niche breadth of species resulting in wider distributions (Bolnick et al., 2011). Thus, we can hypothesize the fact that substantial physiological trait plasticity may have role in conferring the broad ecological amplitude of *R. anthopogon.* Soil physico-chemical properties were also analysed for all the sites along the elevation gradient. The percentage organic matter was considerably low for all the sites and one could suspect the loss of role played by organic matter in improving the soil properties (Kidanemariam et al., 2012). Available nitrogen was consistent across elevations and thus is not a limiting factor of alpine regions (Billings and Mooney, 1968). On the other hand, soil available phosphorus was significantly more in lower elevations in comparison to higher elevations, showing clear disparity among elevations. This may influence the eco-physiology of *R. anthopogon*. In general, changes in soil properties across elevation gradients are thought to be driven by climatic factors (Korner, 2007) and thus require further investigations to understand how these factors may alter soil chemical profiles and ultimately plant growth in the studied region.

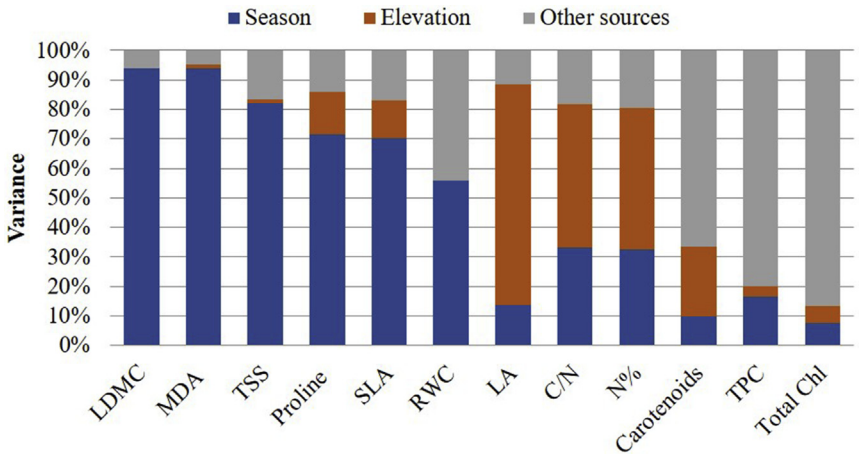

Fig. 5. Variance structure of different eco-physiological variables of *R.anthopogon* depicting individual effect of season, elevation and other sources (between biological replicates and error). RWC = Relative water content; LA = Leaf area; SLA = Specific leaf area; LDMC = Leaf dry matter content; C/N ratio = Carbon nitrogen ratio; N % = Nitrogen percentage; Total Chl = Total chlorophyll; TSS = Total soluble sugars; MDA = Malondialdehyde equivalents; TPC = Total phenolics content.

Table 6
Partitioned variance (%) for eco-physiological variables measured along two effects (season and elevation) for *R.anthopogon*.

S.no	Parameter	Season	Elevation	Other sources
1	LDMC	94.03	0	5.967
2	MDA	93.82	1.33	4.85
3	TSS	82.14	1.217	16.64
4	Proline	71.61	14.29	14.1
5	SLA	70.2	12.77	17.03
6	RWC	55.75	0	44.25
7	Carotenoids	33.14	9.584	66.6
8	LA	13.52	75.03	11.45
9	C/N ratio	33.17	48.72	18.11
10	N%	32.51	48.08	19.41
11	TPC	16.41	3.592	80
12	Total Chl	3.466	22.10	74.44

LDMC = Leaf dry matter content; MDA = Malondialdehyde equivalents; TSS = Total soluble sugars; SLA = Specific leaf area; RWC = Relative water content; LA = Leaf area; C/N ratio = Carbon nitrogen ratio; N% = Nitrogen percentage; TPC = Total phenolics content, Total Chl = Total chlorophyll.

All the trends depicted by two-way ANOVA were also supported by variance partitioning analysis and PCA. The main difference among studied eco-physiological and biogeochemical parameters of *R.anthopogon* is in their response to climatic gradients of elevation and season; the scale and magnitude of this should be known. The distinct clusters visible in PCA biplot were for different sampling periods rather than elevation and hence indicated more pronounced effect of season. Similarly, in variance partitioning analysis also, six traits exhibited more variance fraction due to season as compared to elevation. This indicated that variation in those six leaf traits is largely driven by season rather than elevation. Variance fraction of either season or elevation factor in total variance (100 %) for a particular trait indicates the predominance of that factor in governing the variation in respective trait, whereas, higher fraction of variance due to other sources indicated limited effect of growing season or elevation on that particular trait (Messier et al., 2017). For instance, the majority of the total variance (94.03 %) in LDMC was observed across growing period. This indicated that variation in LDMC is mainly governed by season. Therefore, it suggests that season has more pronounced effect on eco-physiology of *R.anthopogon*.

Plants are being forced to “shift” or “evolve” or “exhibit plasticity” or “go extinct” with climate change (Bellard et al., 2012). Evidences have suggested upward shifts in distribution of species in alpine environments because of global warming (Franca et al., 2016). As the warming due to increase in mean temperatures is mounting in Himalaya in much faster rate in comparison to global average (Salinger et al., 2014), limitations due to low temperature will not persist in the projected climate change scenario. This will lead to upward shift of shrubs in alpine regions. Moreover, with climate change, there will be longer growing season which will further facilitate expansion of this evergreen species to higher elevations (He et al., 2017). Hence, from this study, we infer that the endemic shrub of Himalaya under study has enough physiological and biogeochemical plasticity, and, should in future climates, be advantageous for its survival providing adaptive advantage and expanding its range in near future.

The present work is an attempt to bridge the gap in context to combined effect of elevation and growing season on physiology of broadleaf evergreen shrub species. The results from our study revealed that the “multiple stress factors” varying with elevation and growing season shape plant eco-physiology to maximize fitness. The study demonstrated the role of physiological and biogeochemical traits that enable wide distributions of *R.anthopogon* and tolerate multiple stress factors. We also emphasize on the importance of “seasonal variations coupled with elevation gradient” in research studies related to physiological processes for better understanding of plant adaptive responses and strategies. Also, repeated observations of physiological and biogeochemical trait plasticity across multiple populations will allow us to draw inference about climate change in these natural spatial gradients. The study also suggests high potential of this plant to carry out cold tolerance studies in fragile ecology of Himalaya.

Author contributions

AC conceived the idea of experiment. AC spotted the plant species and its niche and designed the experiment. NR set up the experiment. Ecological sampling was carried out by NR and DT. NR performed all the biochemical and elemental analysis whereas functional traits were recorded by DT. Statistical analysis was done by NR and AC. Variance partitioning analysis was done by DT. NR and AC prepared the draft of manuscript. All authors read and approved the final manuscript.

Declaration of interest

None.

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